

## METHOD OF CANCER TREATMENT BY P53 PROTEIN CONTROL

The present invention relates to a new method for the treatment of cancer. More particularly, it  
5 relates to a method of treating cancer by regulating the cellular levels of the p53 protein. It also relates to vectors for gene therapy which make it possible to regulate the p53 protein, as well as the pharmaceutical compositions containing them.

10 For the past fifteen years, the molecular characterization of oncogenes and of tumour suppressor genes has made it possible to view the process of carcinogenesis in a new light. Thus, the increasingly detailed knowledge of the regulation of these genes and  
15 of the function of the corresponding proteins makes it possible to conceive new therapeutic approaches.

More particularly, the elucidation of the breakdown of the oncogenic and anti-oncogenic proteins represents a major challenge in terms of the fight  
20 against cancer since it presages, in the case of oncogenic proteins, the possibility of accelerating their degradation and therefore of annihilating their action, in the case of tumour suppressors, inhibiting their degradation and therefore increasing their  
25 antiproliferative or anti-tumour effect, in the case of mutated proteins, potentiating their antigenic presentation by molecules of the Major

Histocompatibility Complex and thereby stimulating a tumour-specific immune response, and, in the case where the high expression of the oncogene or of the anti-oncogene is capable of inducing programmed cell death, the possibility of stabilizing these proteins so as to trigger the apoptotic process.

Originally, the p53 protein was classified as a nuclear oncogene since it could, in transfection experiments, extend the life of rodent cells in culture as well as cooperate with activated oncogenes such as ras to transform cells in primary culture. Indeed, the genes used in these first experiments were mutated and led to the expression of variant p53 proteins characterized by a gain in function. Without excluding functions which might still be discovered, it is now known that the p53 protein, at least in its wild-type form, is a transcription factor which negatively regulates growth and cell division and which, in certain situations, is capable of inducing apoptosis (Yonish-Rouach et al., Nature, 352, 345-347, 1991). Given that these properties manifest themselves in a stress situation where the integrity of the cellular DNA is threatened, it has been suggested that p53 is a "guardian of the genome". The presence of mutated p53 proteins in about 40 % of human tumours, all types taken together, reinforces this hypothesis and underlines the probably critical role which mutations of this gene play in the tumour development (for

reviews, see Montenarh, *Oncogene*, 7, 1673-1680, 1992; Oren, *FASEB J.*, 6, 3169-3176, 1992; Zambetti and Levine, *FASEB J.*, 7, 855-865, 1993).

5 The wild-type p53 protein is subject to a complex regulation which involves the control of its synthesis and of its breakdown as well as that of its intracellular location and of its post translational modifications (see the reviews cited above). The wild-type p53 protein is extremely unstable with a half-life  
10 of a few minutes. In contrast, some mutated proteins which accumulate at a high level in tumours have a significantly extended half-life. Little has been clearly established as regards the degradation of p53. Indeed, neither the intracellular sites of degradation,  
15 nor the number and the nature of the catabolic pathways taken, nor the peptide units labelling p53 for its degradation are known. To our knowledge, the only information available relates to the involvement of the enzyme E1 of the ubiquitin cycle under certain  
20 experimental conditions (Ciechanover et al., *Proc. Natl. Acad. Sci. USA* 88, 139-143, 1991; Chowdary et al., *Molec. Cell. Biol.* 14, 1997-2003, 1994). Moreover, it has been shown that certain proteolytic products derived from p53 may be presented in an antigenic  
25 manner.

The present invention results partly from the demonstration that the p53 proteins are substrates for calcium-dependent proteases: the calpains. It results

more particularly from the demonstration that the p53 proteins are degraded specifically by m-calpain or  $\mu$ -calpain. The present invention constitutes the first demonstration of a mechanism for regulating the cellular levels of the p53 proteins and thus offers a new particularly effective and specific approach for modulating the levels of this protein in pathological situations such as especially certain cancers.

In particular, the present invention describes a new approach for the treatment of cancer, based on the use of compounds which modulate the activity of calpains on the p53 proteins, which make it possible either to activate the degradation of the mutated p53 proteins, in order to block their tumorigenic effect and/or to enhance the presentation of immunogenic peptides, or to stabilize the wild-type p53 protein, in order to counterbalance the tumorigenic effect of the mutated proteins expressed in the tumours and/or in order to induce the apoptosis of the tumour cells.

A first subject of the invention therefore consists in the use of a compound capable of modulating the activity of calpain for the preparation of a pharmaceutical composition for the treatment of cancers.

Calpains are ubiquitous enzymes found in most mammalian cells (for a review, see Croall and deMartino, *Physiol. Rev.*, 71, 813-847, 1991). They are

essentially cytoplasmic but they can penetrate into the nucleus by virtue of the destruction of the nuclear envelope during mitosis or following certain stimuli. As indicated above, the proteolytic activity of calpains is dependent on the presence of calcium.

The compounds capable of modulating the activity of calpain for the purposes of the present invention may be of several types.

They may be compounds capable of inhibiting the activity of the calpain on the p53 proteins. These compounds are particularly advantageous since they can be used to inhibit, at least in part, the degradation of the wild-type p53 protein. These compounds therefore make it possible to stabilize intracellularly the wild-type p53 protein and to counterbalance the effect of the mutated forms. Among the inhibitory compounds which can be used within the framework of the invention there may be mentioned the protease inhibitors (leupeptin, aprotinin, PMSF, and the like), the calcium chelators (EGTA, EDTA, and the like) or more specific inhibitors such as calpastatin or any fragment or derivative thereof. Calpastatin is a known inhibitor of the calpains. Its sequence has been described in the prior art (SEQ ID No. 1). A particularly advantageous embodiment of the present invention consists in transferring into the tumours a vector carrying all or part of the sequence encoding calpastatin. This approach is particularly adapted to the treatment of

cancers which always have a wild-type p53 allele, such as colic or bronchial carcinomas for example. Various fragments or derivatives of calpastatin can be used within the framework of the present invention. Such fragments or derivatives may be any molecule obtained from the sequence SEQ ID No. 1 by modification(s) of a genetic and/or chemical nature, preserving the capacity to inhibit, at least in part, the activity of a calpain. Modification of a genetic and/or chemical nature is understood to mean any mutation, deletion, substitution, addition and/or modification of one or more nucleotides. Such modifications may be carried out with various ends, especially that of preparing sequences adapted to expression in a specific type of vector or host, that of reducing the size of the sequence so as to facilitate their cellular penetration, that of increasing the inhibitory activity, or, in a particularly advantageous manner, of increasing the selectivity of the inhibitor towards the activity of the calpains on the degradation of the wild-type p53 protein.

Such modifications may be carried out, for example, by *in vitro* mutagenesis, by introduction of additional constituents or of synthetic sequences, or by deletions or substitutions of the original constituents. When a derivative as defined above is prepared, its activity as inhibitor of the activity of the calpains on p53 proteins can be demonstrated in

several ways, and in particular by bringing into contact the said inhibitor and the various forms of p53 proteins, and then by detecting the degradation products obtained (see Examples 1 to 3). Any other  
5 technique known to persons skilled in the art can obviously be used to this effect.

In a specific embodiment of the present invention, all or part of calpastatin, or a nucleic acid encoding all or part of calpastatin is used as  
10 inhibitor. Still more particularly, a peptide comprising all or part of the sequence SEQ ID No. 1 or of a derivative thereof is used.

As regards more particularly the derivatives, there may be mentioned, by way of example, the compound  
15 of sequence SEQ ID No. 2, which corresponds to a fragment of calpastatin. There is advantageously used any derivative composed of the sequence SEQ ID No. 1 or 2 which is capable of specifically or preferentially inhibiting the degradation of the wild-type p53 protein  
20 by calpain.

The compounds capable of modulating the activity of calpain on the p53 proteins for the purposes of the present invention may also be derivative of calpain capable of specifically or  
25 preferentially degrading the mutated p53 proteins. Such derivatives are also very advantageous since they make it possible to activate the degradation of the mutated p53 proteins, in order to block their tumorigenic

effect and/or to increase the presentation of the immunogenic peptides, without significantly affecting the cellular levels of the wild-type p53 protein. Such derivatives may be obtained from calpain, by structural  
5 modification(s) of a genetic and/or chemical nature. The capacity of the derivatives thus obtained to specifically or preferentially degrade the mutated p53 proteins may then be demonstrated as described in Examples 1 to 3.

10 Preferably, the modulators used within the framework of the invention are proteins or polypeptides, or nucleic acid sequences encoding these polypeptides or proteins. Still more preferably, the modulatory compounds are proteins or polypeptides which  
15 are specific inhibitors of the activity of calpain on the wild-type p53 protein or forms of calpains, modified or otherwise, for specifically degrading the mutated p53 proteins.

In a particularly advantageous manner, the  
20 invention consists in the possibility of bringing about the expression in cancer cells having both a wild-type p53 allele and a mutated p53 allele of nucleic sequences encoding inhibitors of calpain, such as calpastatin or part of calpastatin, or forms of  
25 calpains, modified or otherwise, for specifically degrading the mutated p53 proteins.

The nucleic acid sequence used within the framework of the present invention may be administered

as such, in the form of naked DNA according to the technique described in Application WO 90/11092. It can also be administered in a form complexed, for example, with DEAE-dextran (Pagano et al., J. Virol. 1 (1967) 891), with nuclear proteins (Kaneda et al., Science 243 (1989) 375), with lipids (Felgner et al., PNAS 84 (1987) 7413), in the form of liposomes (Fraley et al., J. Biol. Chem. 255 (1980) 10431), and the like.

Preferably, the sequence used within the framework of the invention forms part of a vector. The use of such a vector indeed makes it possible to improve the administration of the nucleic acid into the cells to be treated, and also to increase its stability in the said cells, which makes it possible to obtain a lasting therapeutic effect. Furthermore, it is possible to introduce several nucleic acid sequences into the same vector, which also increases the efficacy of the treatment.

The vector used may be of various origin, as long as it is capable of transforming animal cells, preferably human cancer cells. In a preferred embodiment of the invention, a viral vector is used which may be chosen from adenoviruses, retroviruses, adeno-associated viruses (AAV) or the herpes virus.

In this regard, the subject of the present invention is any recombinant virus comprising, inserted into its genome, a nucleic acid encoding a compound capable of modulating the activity of calpain.

Preferably, the viruses used within the framework of the invention are defective, that is to say that they are incapable of replicating autonomously in the infected cell. Generally, the genome of the defective viruses used within the framework of the present invention therefore lacks at least the sequences necessary for the replication of the said virus in the infected cell. These regions may be either removed (completely or in part), or made nonfunctional, or substituted by other sequences and especially by the sequence encoding the modulator of the calpains. Preferably, the defective virus retains, nevertheless, the sequences of its genome which are necessary for the encapsidation of the viral particles.

As regards more particularly adenoviruses, various serotypes, whose structure and properties vary somewhat, have been characterized. Among these serotypes, the use of the type 2 or 5 human adenoviruses (Ad 2 or Ad 5) or of the adenoviruses of animal origin (see application FR 93 05954) is preferred within the framework of the present invention. Among the adenoviruses of animal origin which can be used within the framework of the present invention, there may be mentioned adenoviruses of canine, bovine, murine (example: MVA1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or alternatively simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus,

or more preferably a CAV2 adenovirus [Manhattan strain or A26/61 (ATCC VR-800) for example]. Preferably, adenoviruses of human or canine or mixed origin are used within the framework of the invention.

5                    Preferably, the defective adenoviruses of the invention comprise the ITRs, a sequence allowing the encapsidation and the sequence encoding the modulator of the calpains. Still more preferably, in the genome of the adenoviruses of the invention, the E1 gene and  
10                    at least one of the genes E2, E4, L1-L5 are nonfunctional. The viral gene considered can be rendered non-functional by any technique known to persons skilled in the art, and especially by total  
15                    suppression, by substitution or partial deletion, or by addition of one or more bases in the gene(s) considered. Such modifications can be obtained in vitro (on the isolated DNA) or in situ, for example by means of genetic engineering techniques, or alternatively by  
20                    treating with mutagenic agents.

20                    The defective recombinant adenoviruses according to the invention can be prepared by any technique known to persons skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be  
25                    prepared by homologous recombination between an adenovirus and a plasmid carrying, inter alia, the DNA sequence encoding the modulator of the calpains. The homologous recombination occurs after co-transfection

of the said adenoviruses and plasmid into an appropriate cell line. The cell line used should preferably (i) be transformable by the said elements, and (ii) contain the sequences capable of complementing the defective adenovirus genome part, preferably in integrated form in order to avoid risks of recombination. As an example of a cell line, there may be mentioned the human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains especially, integrated in its genome, the left hand part of the genome of an Ad5 adenovirus (12 %). Strategies for constructing vectors derived from adenoviruses have also been described in Applications Nos. FR 93 05954 and FR 93 08596.

Next, the adenoviruses which have multiplied are recovered and purified according to conventional molecular biology techniques as illustrated in the examples.

As regards the adeno-associated viruses (AAV), they are relatively small DNA viruses which become integrated into the genome of the cells which they infect, in a stable and site-specific manner. They are capable of infecting a broad spectrum of cells, without inducing any effect on cell growth, morphology or differentiation. Moreover, they do not seem to be involved in pathologies in man. The genome of the AAVs has been cloned, sequenced and characterized. It comprises about 4700 bases and contains, at each end,

an inverted repeat region (ITR) of about 145 bases which serves as replication origin for the virus. The remainder of the genome is divided into 2 essential regions carrying the encapsidation functions: the left hand part of the genome, which contains the rep gene involved in the viral replication and the expression of the viral genes; the right hand part of the genome, which contains the cap gene encoding the virus capsid proteins.

10                   The use of vectors derived from AAVs for the transfer of genes in vitro and in vivo has been described in the literature (see especially WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). These applications describe various  
15                   constructs derived from AAVs, from which the rep and/or cap genes are deleted and replaced by a gene of interest, and their use for the transfer in vitro (on cells in culture) or in vivo (directly in an organism) of the said gene of interest. The defective recombinant  
20                   AAVs according to the invention can be prepared by co-transfection, into a cell line infected by a human helper virus (for example an adenovirus), of a plasmid containing the sequence encoding the modulator of the calpains bordered by two AAV inverted repeat regions  
25                   (ITR), and of a plasmid carrying the AAV encapsidation genes (rep and cap genes). The recombinant AAVs produced are then purified by conventional techniques.

As regards the herpes viruses and the

retroviruses, the construction of recombinant vectors has been widely described in the literature: see especially Breakfield et al., New Biologist 3 (1991) 203; EP 453242, EP 178220, Bernstein et al. Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689, and the like.

For carrying out the present invention, it is most particularly advantageous to use a defective recombinant retrovirus or adenovirus. These vectors indeed have particularly advantageous properties for the transfer of genes into tumour cells.

Advantageously, in the vectors of the invention, the sequence encoding the modulator of the calpains is placed under the control of signals allowing its expression in tumour cells. Preferably, these are heterologous expression signals, that is to say signals different from those which are naturally responsible for the expression of the modulator. They may be in particular sequences responsible for the expression of other proteins, or synthetic sequences. In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, they may be promoter sequences derived from the genome of a virus, including the virus used. In this regard, the E1A, MLP, CMV, RSV-LTR promoters and the like may be mentioned for example. In addition, these expression sequences

may be modified by addition of activating or regulatory sequences or of sequences allowing a tissue-specific expression. It may indeed be particularly advantageous to use expression signals which are active specifically or predominantly in tumour cells, so that the DNA sequence is expressed or produces its effect only when the virus has effectively infected a tumour cell.

In a specific embodiment, the invention relates to a defective recombinant virus comprising a cDNA sequence encoding a modulator of the calpains under the control of a viral promoter, preferably chosen from the RSV-LTR and the CMV promoter.

Still in a preferred embodiment, the invention relates to a defective recombinant virus comprising a DNA sequence encoding a modulator of the calpains under the control of a promoter allowing predominant expression in tumour cells.

The expression is considered to be predominant for the purposes of the invention when, even if a residual expression is observed in other cell types, the expression levels are greater in the tumour cells.

The present invention also relates to any pharmaceutical composition comprising one or more defective recombinant viruses as described above. These pharmaceutical compositions may be formulated for administrations via the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous,

intraocular or transdermal route and the like.

Preferably, the pharmaceutical compositions of the invention contain a vehicle pharmaceutically acceptable for an injectable formulation, especially for a direct  
5 injection into the patient's tumour. This may be in particular isotonic sterile solutions, or dry, especially freeze-dried, compositions which, upon addition, depending on the case, of sterile water or of physiological saline, allow the preparation of  
10 injectable solutions. Direct injection into the patient's tumour is advantageous because it makes it possible to concentrate the therapeutic effect at the level of the affected tissues.

The doses of defective recombinant virus  
15 which are used for the injection may be adapted according to various parameters, and especially according to the viral vector, the mode of administration used, the relevant pathology or alternatively the desired duration of the treatment. In  
20 general, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between  $10^4$  to  $10^{14}$  pfu/ml, and preferably  $10^6$  to  $10^{10}$  pfu/ml. The term pfu ("plaque forming unit") corresponds to the infectivity of a virus solution, and  
25 is determined by infecting an appropriate cell culture and measuring, generally after 48 hours, the number of plaques of infected cells. The techniques for determining the pfu titre of a viral solution are well

documented in the literature. As regards the retroviruses, the compositions according to the invention may directly comprise the producing cells, for their implantation.

5           The present invention is particularly adapted to the treatment of cancers in which the mutated forms of p53 are observed. More specifically, the present invention is particularly advantageous for the treatment of cancers in which the wild-type and mutated  
10 alleles of p53 are present. Such cancers are especially colorectal cancer, breast cancer, lung cancer, gastric cancer, oesophageal cancer, B lymphomas, ovarian cancer, cancer of the bladder and the like.

          The present invention will be more fully  
15 described with the aid of the following Examples which should be considered as illustrative and nonlimiting.

#### Legend to the Figures

Figure 1: Study of the regulation of the p53 protein by calpain. The reaction is carried out in a final volume  
20 of 30  $\mu$ l, of which 1 comes from the translation mixture. Line 1: T0; line 2: 30 min in the presence of 1 mM Calcium + 20  $\mu$ g/ml Calpain; line 4: 30 min in the presence of 1 mM Calcium + 20  $\mu$ g/ml Calpain + 0.5 mg/ml calpastatin; line 5: 30 min in the presence of 1 mM  
25 Calcium + 20  $\mu$ g/ml Calpain + 10 mM EGTA; line 6: PBS; line 7: PBS + calcium; line 8: PBS + calpastatin.

#### General molecular biology techniques

The methods conventionally used in molecular

biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in caesium chloride gradient, agarose or acrylamide gel electrophoresis, purification of DNA fragments by electroelution, phenol or phenol-chloroform extraction of proteins, ethanol or isopropanol precipitation of DNA in saline medium, transformation in *Escherichia coli* and the like, are well known to persons skilled in the art and are widely described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

The pBR322 and pUC type plasmids and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments can be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol and then incubated in the presence of phage T4 DNA ligase (Biolabs) according to the recommendations of the supplier.

The filling of the protruding 5' ends can be performed with the Klenow fragment of *E. coli* DNA polymerase I (Biolabs) according to the specifications of the supplier. The destruction of the protruding 3' ends is performed in the presence of phage T4 DNA

polymerase (Biolabs) used according to the recommendations of the manufacturer. The destruction of the protruding 5' ends is performed by a controlled treatment with S1 nuclease.

5                    Site-directed mutagenesis in vitro by synthetic oligodeoxynucleotides can be performed according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

10                    The enzymatic amplification of the DNA fragments by the so-called PCR technique [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be performed using  
15 a DNA thermal cycler (Perkin Elmer Cetus) according to the specifications of the manufacturer.

                  The verification of the nucleotide sequences can be performed by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-  
20 5467] using the kit distributed by Amersham.

### Examples

#### Example 1

                  This example shows that the addition of m-calpain to rabbit reticulocyte lysate induces the  
25 degradation of the wild-type p53 protein as well as that of certain mutated forms. This example also shows that inhibitors of calpains are capable of inhibiting the degradation of p53 and therefore of modulating the

activity of this protein.

1.1. Demonstration of the degradation: mouse and human wild-type p53 proteins as well as various mutated p53 proteins (human proteins C273, H273, H175, I247) were translated in the rabbit reticulocyte lysate. The proteins thus obtained are resistant to any degradation, even in the presence of a high concentration of calcium (cofactor essential for the calpains). The addition of bovine m-calpain (Sigma) to the reticulocyte lysate in the presence of calcium led to the rapid disappearance of the neosynthesized proteins and the appearance of proteolytic fragments which are resolvable by electrophoresis. The degradation resistance of other proteins such as dihydrofolate reductase or glyceraldehyde-3-phosphate dehydrogenase under the same experimental conditions indicates the substrate specificity of the reaction.

1.2. Use of inhibitors of calpain for modulating the levels of p53 proteins: in the above Example 1.1., it was shown that the addition of m-calpain induced degradation of the p53 proteins. In this example, in addition to m-calpain, various compounds were introduced into the medium in order to test their capacity to inhibit the activity of calpain. The results obtained show that the addition of a calcium chelator (EGTA) as well as of a peptide which is a specific inhibitor of the calpains (derivative of a physiological inhibitor, calpastatin; Maki et al., J.

Biol. Chem., 254, 18866-18869, 1989) are capable of inhibiting the degradation of the p53 proteins which is induced by the exogenous calpain.

#### Example 2

5           In the preceding example, it was shown that the addition of exogenous calpain to a solution of p53 proteins brought about their degradation. This example shows that the degradation of the wild-type p53 protein as well as that of certain mutated forms may be induced  
10 by the endogenous calpains in cytoplasmic extracts. This example also shows that inhibitors of the calpains are capable, in the presence of endogenous calpain, of inhibiting the degradation of p53 and therefore of modulating the activity of this protein.

15           2.1. Degradation by the endogenous calpains: mouse and human wild-type p53 proteins, as well as certain mutated forms (cf Example 1) were translated in the reticulocyte lysate and were then incubated in the presence of cytoplasmic extracts of Daudi or Jurkat  
20 human lymphoblastoid cells. The cytoplasmic extracts were prepared in the following manner: the cells (available at the ATCC) were cultured in DMEM medium supplemented with 10 % foetal calf serum. The cells were then harvested, washed in PBS buffer and then  
25 incubated for 5 min in a detergent-free hypotonic lysis buffer (HEPES 20 mM, pH 7.5; KOAc 10 mM; MgOAc 1.5 mM; 2 ml per  $5 \times 10^8$  cells). The lysis was completed using a Dounce homogenizer and then checked under a

microscope. The nuclei were then removed by centrifugation at 2000 g for 5 min, and the supernatants were centrifuged at 10,000 g for 1 hour (Beckman SW60). The cytoplasmic extracts were then  
5 aliquoted in an amount of 5 to 12 mg/ml.

When the lysate of reticulocytes was incubated in the presence of cytoplasmic extracts, in the absence of calcium, no degradation was observed. On the other hand, in the presence of calcium, a very  
10 rapid degradation of the p53 proteins was observed, with the appearance of a characteristic proteolytic product profile similar to that obtained in Example 1. This experiment indeed shows that the p53 proteins are degraded by the endogenous calpains.

15 2.2. Use of calpain inhibitors to modulate the levels of p53 proteins: the chelation of calcium by EGTA, as well as the use of a whole range of protease inhibitors (leupeptin, aprotinin, soybean trypsin inhibitor and PMSF) and especially the peptide  
20 calpastatin show that the degradation of these proteins is dependent on the calpains of the cytoplasmic extract, and that various compounds capable of modulating the activity of the calpains may be used to regulate the p53 protein levels.

25 Example 3

This example demonstrates that the mouse and human wild-type p53 proteins are direct substrates for the calpains in the cytoplasmic extracts.

Examples 1 and 2 show that the calpains can induce the degradation of p53 in complex reaction mixtures. These experiments do not exclude, however, that under the conditions used, the calpains activate secondary proteases which are those which actually act on p53. In this example, the following experiment was conducted: (1) the mouse and human wild-type p53 proteins neosynthesized in the rabbit reticulocyte lysate were incubated for 30 minutes in the presence of a cytoplasmic extract of Daudi cells as well as in the presence of calcium to activate the calpains as in Example 2, (2) p53 protein was then added to the reaction mixture and the reaction was continued for 30 minutes under conditions permissive (same reaction conditions) or otherwise (addition either of EGTA to chelate the calcium, or of calpastatin peptide) for the calpains. In the presence of calcium, the newly added p53 protein is completely degraded, indicating that the protease activity is functional throughout the experiment. When the calpains are inhibited by the presence of EGTA or, more significantly, of the calpastatin peptide, the newly added p53 protein is, on the other hand, no longer degraded. This latter observation therefore excludes the possibility that in the first part of the experiment, the calpains induced a second protease responsible for the degradation of p53 (Figure 1).

Example 4

This example describes the construction of a recombinant adenovirus comprising a nucleic acid sequence encoding calpastatin. This adenovirus is constructed by homologous recombination between the defective adenovirus Ad-d11324 and a plasmid carrying the sequence SEQ ID No. 1 under the control of the RSV promoter.

## 4.1. Construction of the plasmid SEQ ID No. 1

The plasmid SEQ ID No. 1 comprises the sequence encoding calpastatin under the control of the RSV-LTR promoter, as well as regions of the adenovirus which allow homologous recombination. It is constructed by inserting the sequence SEQ ID No. 1 into the plasmid pAd.RSV $\beta$ gal. The plasmid pAd.RSV $\beta$ Gal contains, in the 5'  $\rightarrow$  3' orientation,

- the PvuII fragment corresponding to the left hand end of the Ad5 adenovirus comprising: the ITR sequence, the replication origin, the encapsidation signals and the enhancer E1A;

- the gene encoding  $\beta$ -galactosidase under the control of the RSV promoter (Rous sarcoma virus),

- a second fragment of the Ad5 adenovirus genome which allows homologous recombination between the plasmid pAd.RSV $\beta$ Gal and the adenovirus d1324. The plasmid pAd.RSV $\beta$ Gal has been described by Stratford-Perricaudet et al. (J. Clin. Invest. 90 (1992) 626).

#### 4.2. Construction of the recombinant adenovirus

The vector described in 4.1. is linearized and cotransfected with a deficient adenoviral vector into the helper cells (line 293) providing in trans the functions encoded by the adenovirus E1 regions (E1A and E1B).

More specifically, the recombinant adenovirus is obtained by homologous recombination in vivo between the mutant adenovirus Ad-dl1324 (Thimmappaya et al., Cell 31 (1982) 543) and the vector described in Example 4.1., according to the following procedure: the plasmid SEQ ID No. 1 and the adenovirus Ad-dl1324, linearized by the enzyme ClaI, are cotransfected into the line 293 in the presence of calcium phosphate, so as to allow the homologous recombination. The recombinant adenoviruses thus generated are then selected by plaque purification. After isolation, the recombinant adenovirus DNA is amplified in the cell line 293, leading to a culture supernatant containing the unpurified recombinant defective adenovirus having a titre of about  $10^{10}$  pfu/ml.

The viral particles are purified by centrifugation on a caesium chloride gradient according to known techniques (see especially Graham et al., Virology 52 (1973) 456). The adenovirus obtained may be stored at  $-80^{\circ}\text{C}$  in 20 % glycerol.